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ASTROCYTES REGULATE α -SECRETASE-CLEAVED SOLUBLE AMYLOID PRECURSOR PROTEIN SECRETION IN NEURONAL CELLS: INVOLVEMENT OF GROUP IIA SECRETORY PHOSPHOLIPASE A₂

X. YANG^a, W. SHENG^b, D. M. RIDGLEY^c, M. A. HAIDEKKER^d, G. Y. SUN^b, and J. C. LEE^{c,e,*}

^aHope Center for Neurological Disorders and Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110, United States

^bDepartment of Biochemistry, University of Missouri, Columbia, MO 65211, United States

^cDepartment of Bioengineering, University of Missouri, Columbia, MO 65211, United States

^dCollege of Engineering, Driftmier Engineering Center, University of Georgia, Athens, GA 30602, United States

^eDepartment of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607, United States

Abstract

Astrocytes are major supportive cells in brains with important functions including providing nutrients and regulating neuronal activities. In this study, we demonstrated that astrocytes regulate amyloid precursor protein (APP) processing in neuronal cells through secretion of group IIA secretory phospholipase A₂ (sPLA₂-IIA). When astrocytic cells (DITNC) were mildly stimulated with the pro-inflammatory cytokines, such as TNF α and IL-1 β , sPLA₂-IIA was secreted into the medium. When conditioned medium containing sPLA₂-IIA was applied to human neuroblastoma (SH-SY5Y) cells, there was an increase in both cell membrane fluidity and secretion of α -secretase-cleaved soluble amyloid precursor protein (sAPP α). These changes were abrogated by KH064, a selective inhibitor of sPLA₂-IIA. In addition, exposing SH-SY5Y cells to recombinant human sPLA₂-IIA also increased membrane fluidity, accumulation of APP at the cell surface, and secretion of sAPP α , but without altering total expressions of APP, α -secretases and β -site APP cleaving enzyme (BACE1). Taken together, our results provide novel information regarding a functional role of sPLA₂-IIA in astrocytes for regulating APP processing in neuronal cells.

Keywords

cytokine; astrocytes; sPLA₂-IIA; sAPP α ; membrane fluidity; SH-SY5Y

*Corresponding author. Address: 240 Agricultural Engineering Building, University of Missouri, Columbia, MO 65211, United States. Tel: +1-573-884-3686; fax: +1-573-882-1115. leejam@missouri.edu (J. C. Lee).

CONFLICT OF INTEREST DISCLOSURE

The authors have no conflict of interest to declare.

INTRODUCTION

Astrocytes are multifunctional house-keeping cells playing important roles in regulating the environment within which neurons function (Nedergaard et al., 2003; Pereira and Furlan, 2010). These cells are known to control local ion and pH homeostasis, provide glucose, deliver metabolic substrates, maintain blood–brain barrier, and remove neuronal waste. In many instances, astrocytes modulate neuronal activities by means of release of signaling molecules, including glutamate, D-serine, and adenosine. They also regulate neurogenesis, synaptogenesis, and synaptic activity, although the underlying mechanism(s) remain to be elucidated.

Phospholipases A₂ (PLA₂s) are ubiquitous enzymes responsible for maintenance of phospholipid homeostasis in cell membranes. There are three major types of PLA₂s, namely, cytosolic PLA₂ (cPLA₂), calcium-independent PLA₂ (iPLA₂), and secretory PLA₂ (sPLA₂) (Sun et al., 2004). sPLA₂ are low molecular weight proteins with more than 12 subtypes present in a wide variety of cells and venoms. One of the subtypes, the group IIA sPLA₂ (sPLA₂-IIA) has been identified in astrocytes (Lin et al., 2004) and is known to modulate neuronal functions, including calcium influx and apoptosis (Morioka et al., 2002; Yagami et al., 2003, 2014). sPLA₂-IIA also has antibacterial functions (Harwig et al., 1995; Weinrauch et al., 1996) and increased production has been linked to inflammatory diseases, including rheumatoid arthritis and Alzheimer's disease (AD) (Hurt-Camejo et al., 2001; Sun et al., 2004, 2007; Jia et al., 2005; Moses et al., 2006). In cell culture systems, upregulation of sPLA₂-IIA mRNA expression has been shown in astrocytes stimulated with pro-inflammatory cytokines, processes that activate the NF-κB pathway (Li et al., 1999; Wang and Sun, 2000; Jensen et al., 2009). Using non-specific PLA₂ inhibitors, Cho et al. (2006) showed that PLA₂s affect the proteolytic process of amyloid precursor protein (APP), which produces either neurotoxic amyloid-β peptide (Aβ) (Vassar, 2004) or neurotrophic and neuroprotective α-secretase-cleaved soluble APP (sAPP_α) (Esch et al., 1990; Allinson et al., 2003; Thornton et al., 2006). In our previous study, we showed that group III sPLA₂ (sPLA₂-III) from bee venom increased sAPP_α production through altering membrane fluidity (Yang et al., 2010). sPLA₂-IIA has been shown to effect various processes associated with neuroinflammatory diseases such as arthritis and atherosclerosis (Touqui and Alaoui-El-Azher, 2001; Niessen et al., 2003). Furthermore, sPLA₂-IIA has been shown to be upregulated in the astrocytes of AD patients which directly implicates sPLA₂-IIA in AD pathogenesis (Moses et al., 2006). Taken together, the aforementioned processes of sPLA₂-IIA and its increased presence around Aβ plaque sites within the brains of AD patients need to be further understood to offer insights into AD etiology.

In this study, we explored the effects of sPLA₂-IIA secreted from cytokine-stimulated immortalized rat astrocytes (DITNC) on membrane integrity, APP processing and sAPP_α secretion in human neuroblastoma cells (SH-SY5Y). Specifically, we demonstrated that TNF_α and IL-1 β can induce DITNC cells to secrete sPLA₂-IIA into the conditioned medium, which in turn, altered membrane fluidity and sAPP_α secretion in differentiated SH-SY5Y cells. In order to further verify the role of sPLA₂-IIA in sAPP_α secretion, we tested the effects of a recombinant human sPLA₂-IIA on sAPP_α secretion, membrane fluidity, the recruitment of APP to the cell surface, and the expressions of α-secretases, and β-site APP

cleaving enzyme (BACE1) in SH-SY5Y cells. AD is associated with chronic inflammation and this study demonstrates that upregulation of sPLA₂-IIA in astrocytes influences neuronal membrane properties and APP metabolism. Here we present novel information to better understand the role of cytokine-stimulated astrocyte secretion of sPLA₂-IIA and its subsequent effects in neurons.

EXPERIMENTAL PROCEDURES

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) with high glucose, DMEM/F12 medium (1:1), Ham's F-12 medium, fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Invitrogen (Carlsbad, CA, USA). Recombinant human sPLA₂-IIA was from BioVendor (Candler, NC, USA). Selective sPLA₂-IIA inhibitor (KH064) was from EMD Biosciences (San Diego, CA, USA). Cytokines including TNF α and IL-1 β were from R&D Systems (Minneapolis, MN, USA). Phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO) and all-*trans* retinoic acid (RA) were from Sigma-Aldrich (St. Louis, MO, USA). Farnesyl-(2-carboxy-2-cyanovinyl)-julolidine (FCVJ) was from Dr. Haidekker's Laboratory (University of Georgia) (Nipper et al., 2008).

Cell culture

SH-SY5Y cells (1.0×10^5 cells/well) (ATCC, Manassas, VA, USA) were seeded into 12-well plates or 60-mm dishes (1.0×10^6 cells/dish) and were cultured in DMEM/F12 medium (1:1) containing 10% FBS. For differentiation, SH-SY5Y cells were exposed to 10 μ M RA for 6 days with changes of fresh culture medium every 2 d. The rat immortalized astrocytes (DITNC) were obtained from ATCC and cultured in DMEM medium supplemented with 10% FBS. All cells were maintained at 37 °C in a 5% CO₂ humidified incubator.

Cell viability by MTT test

Cell viability was determined by MTT reduction. Briefly, differentiated SH-SY5Y cells cultured in 12-well plates were treated with different concentrations of sPLA₂-IIA. After treatment, the medium was removed and 1 ml of MTT reagent (0.5 mg/ml) in DMEM was added into each well. Cells were incubated for 4 h at 37 °C and after dissolving formazan crystals with DMSO, absorbance at 540 nm was measured.

Characterization of membrane fluidity by fluorescence microscopy of FCVJ-labeled cells

A fluorescent molecular rotor, farnesyl-(2-carboxy-2-cyanovinyl)-julolidine (FCVJ) was used to measure the relative membrane fluidity in SH-SY5Y cells. FCVJ was designed to be a membrane-compatible fluorescent molecular rotor (Haidekker et al., 2001) with the quantum yield strongly dependent on the local free volume. A higher fluorescent intensity of FCVJ reflects the intramolecular-rotational motions being restricted by a smaller local free volume, indicating a more viscous membrane. On the other hand, a lower fluorescent intensity of FCVJ reflects a lower viscous and a more fluidized membrane. Previously, we have verified the application of FCVJ for measuring membrane fluidity by comparing results obtained using FCVJ with those from the technique of fluorescence recovery after

photobleaching (FRAP) (Nipper et al., 2008). In this study, we adapted the protocol from Haidekker et al. (2001) to fluorescently label cells with FCVJ. Briefly, after treatment with sPLA₂-IIA or conditioned medium from DITNC cells, SH-SY5Y cells were washed with PBS and incubated in DMEM containing 20% FBS and 1 μ M FCVJ for 20 min. Excess FCVJ was removed by washing cells with PBS three times. Fluorescent intensity measurements were performed at room temperature using a Nikon TE-2000 U fluorescence microscope with an oil immersion 60 $\times$ objective lens. Images were acquired using a cooled-CCD camera controlled by a computer running a MetaVue imaging software (Universal Imaging, PA, USA). The fluorescent intensities of FCVJ per cell area were measured. Background subtraction was done for all images prior to data analysis.

Treatment of SH-SY5Y cells with conditioned medium from DITNC astrocytes

DITNC astrocytes were exposed to cytokines (TNF α and IL-1 β , 10 ng/ml) for 8 h. Cytokines were then removed, and the cells were incubated in serum-free medium for another 40 h. The same volume of conditioned medium from control and cytokine-stimulated cells was used for Western blot analysis of sPLA₂-IIA. Alternatively, the conditioned medium from control and cytokine-stimulated DITNC cells were applied to SH-SY5Y cells for 24 h. In order to demonstrate the effects of sPLA₂-IIA in the conditioned medium on sAPP α secretion and membrane fluidity in SH-SY5Y cells, (S)-5-(4-(benzyloxy)phenyl)-4-(7-phenylheptanamido)pentanoic acid (KH064), a selective sPLA₂-IIA inhibitor (1 μ M), was added to the conditioned medium prior to applying to SH-SY5Y cells. KH064 has been used as a potent sPLA₂-IIA inhibitor previously (Gregory et al., 2006).

Western blot analysis of sPLA₂-IIA released from DITNC astrocytes and sAPP α released from SH-SY5Y cells

After treating DITNC astrocytes with cytokines, conditioned medium was used for Western blot analysis for sPLA₂-IIA. After treating SH-SY5Y cells with conditioned media, or recombinant human sPLA₂-IIA, culture media were collected for measurement of sAPP α . β -actin in cell lysate was the internal standard. The collected medium was centrifuged at 12,000g for 5 min to remove cell debris, and the same volume of medium from each sample (e.g., 40 μ l) was diluted with Laemmli buffer, boiled for 5 min, subjected to electrophoresis in 7.5% SDS–polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and were incubated overnight at 4 $^{\circ}$ C in 3% (w/v) bovine serum albumin (BSA) with 0.02% (w/v) sodium azide in TBST with a 6E10 monoclonal antibody (1:1000 dilution; Millipore, Billerica, MA) that recognizes residues 1–17 of the A β domain of human sAPP α or with anti-sPLA₂-IIA rabbit polyclonal antibody (1:1000; BioVendor, Candler, NC). Membranes were washed three times during a 15-min period with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h. After washing with TBST for three times, the membrane was subjected to SuperSignal West Pico Chemiluminescent detection reagents from Pierce (Rockford, IL, USA) to visualize bands. The protein bands detected on X-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad).

Western blot analysis of APP, ADAM9, ADAM10, ADAM17 and BACE1 in SH-SY5Y cells

After treatments, the protein concentration of the SH-SY5Y cell lysate was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction. Equivalent amounts of protein from each sample (e.g., 30 µg) was diluted with Laemmli buffer, boiled for 5 min, subjected to electrophoresis in 7.5% SDS–polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and were incubated overnight at 4 °C in 3% (w/v) BSA with 0.02% (w/v) sodium azide in TBST with 6E10 monoclonal antibody, anti-ADAM9 antibody (1:1000 dilution; Abcam, Cambridge, MA, USA), anti-ADAM10 antibody (1:1000 dilution; Millipore, Billerica, MA, USA), anti-ADAM17 antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-BACE1 antibody (1:1000 dilution; Sigma–Aldrich, St. Louis, MO, USA). Membranes were washed three times during a 15-min period with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h. After washing with TBST for three times, the membrane was subjected to SuperSignal West Pico Chemiluminescent detection reagents from Pierce (Rockford, IL, USA) to visualize bands. The protein bands detected on X-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad).

Immunofluorescent staining and assessment of APP at the cell surface of SH-SY5Y cells

SH-SY5Y cells were plated onto cover slips and after differentiation and treatments, they were fixed in PBS containing 4% paraformaldehyde without prior permeabilization with detergent in a similar manner as previously reported (Yang et al., 2009, 2010). After washing three times with PBS, nonspecific binding of antibodies was blocked by 5% goat serum for 1 h at room temperature. Cells were then incubated overnight at 4 °C in 3% goat serum with anti-APP mouse antibody (1:200 dilution; assay designs, Ann Arbor, MI, USA) that recognizes the N-terminus of APP. The cover slips were washed with PBS and incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody (1:400, Santa Cruz, CA, USA) and washed with PBS. Cover slips were then mounted and fluorescent intensity measurements were performed at room temperature using the Nikon TE-2000 U fluorescence microscope and oil immersion 60× objective lens. Images were acquired using a CCD camera controlled by a computer running a MetaVue imaging software (Universal Imaging, PA, USA). The fluorescent intensities per cell area were measured. Background subtraction was done for all images prior to data analysis.

Quantification of Aβ_{1–42}

After treatments, culture medium and cell lysates were collected, supplemented with protease inhibitor cocktail and conditioned medium was centrifuged at 12,000g for 5 min at 4 °C to remove cell debris. An aliquot (100 µl) of the supernatant was used for Aβ_{1–42} quantification using an ELISA kit specific for Aβ_{1–42} (Invitrogen, Carlsbad, CA, USA) following manufacturer's recommendation. According to the instruction manual, Aβ_{1–12},

A β ₁₋₂₀, A β ₁₂₋₂₈, A β ₂₂₋₃₅, A β ₁₋₄₀, A β ₁₋₄₃, A β ₄₂₋₁ and APP show no cross-reactivity. The minimum detectable dose of A β ₁₋₄₂ is <1.0 pg/ml. The level of A β ₁₋₄₂ in each sample was measured in duplicates and expressed in pg/ml.

Statistical analysis

Data are presented as mean $\pm$ SD from at least three independent experiments. Comparison between two groups was made with student's *t* test. Comparisons of more than two groups were made with an one-way ANOVA, followed by *Bonferroni's* post hoc tests. Values of *p* < 0.05 are considered to be statistically significant.

RESULTS

Secretion of sPLA₂-IIA from cytokine-stimulated DITNC astrocytes

It has been shown that when stimulated by cytokines such as TNF α and IL-1 β , DITNC astrocytes showed increased mRNA expression of sPLA₂-IIA (Li et al., 1999; Wang and Sun, 2000; Jensen et al., 2009). In this study, we first determined whether stimulation of DITNC astrocytes with TNF α and IL-1 β induced sPLA₂-IIA secretion. Western blot analysis showed that no sPLA₂-IIA was detected in the culture medium of non-stimulated (control) cells whereas cytokines stimulated DITNC astrocytes to secrete sPLA₂-IIA into the conditioned medium (Fig. 1).

Conditioned medium from cytokine-stimulated DITNC astrocytes increased membrane fluidity and sAPP α secretion in SH-SY5Y cells

Since PLA₂s have been shown to increase sAPP α secretion in SH-SY5Y cells (Cho et al., 2006; Yang et al., 2010), it is reasonable to hypothesize a role for sPLA₂-IIA on mediating increase in sAPP α secretion in SH-SY5Y cells. To test this hypothesis, we applied the conditioned medium from control and cytokine-stimulated DITNC cells to SH-SY5Y cells. As indicated by a lower FCVJ intensity, the conditioned medium containing sPLA₂-IIA from cytokine-stimulated DITNC astrocytes caused an increase in membrane fluidity in SH-SY5Y cells (Fig. 2A). The conditioned medium also promoted sAPP α secretion in SH-SY5Y cells (Fig. 2B). Furthermore, there is a significant reduction of membrane fluidity and sAPP α secretion when a selective inhibitor for sPLA₂-IIA, KH064, is introduced to the stimulated and non-stimulated conditioned media (Fig. 2). sPLA₂-IIA inhibitor alone did not cause any effects on membrane fluidity and sAPP α secretion (Fig. 2). The results indicate that sPLA₂-IIA from stimulated DITNC cells plays an important role in SH-SY5Y cell membrane fluidity and sAPP α secretion.

Exogenous sPLA₂-IIA increased membrane fluidity and sAPP α secretion in SH-SY5Y cells

To further test the ability for sPLA₂-IIA to alter membrane fluidity and sAPP α secretion, we investigated the effects of recombinant human sPLA₂-IIA. First, we examined the viability of SH-SY5Y cells in response to different doses of sPLA₂-IIA using the MTT test. Results in Fig. 3 show a dose-dependent decrease in cell viability upon exposing SH-SY5Y cells to sPLA₂-IIA for 24 h. The results show that there is no significant decrease in cell viability with a 50 ng/ml dose while a 100 ng/ml dose will yield ~75% SH-SY5Y cell viability. All of

the subsequent experiments were performed with both 50 and 100 ng/ml sPLA₂-IIA concentrations to assess how sPLA₂-IIA affects SH-SY5Y cells.

The recombinant human sPLA₂-IIA was also used to test membrane fluidity. As shown in Fig. 4A, SH-SY5Y cells exhibited a lower fluorescent intensity of FCVJ after treatment with sPLA₂-IIA, as compared with control, suggesting that sPLA₂-IIA action can increase membrane fluidity in SH-SY5Y cells. Together, these data suggest that sPLA₂-IIA increased sAPP_α through its action to increase membrane fluidity. Western blot analysis showed that sPLA₂-IIA (50 and 100 ng/ml) increased sAPP_α secretion in SH-SY5Y cells (Fig. 4B). Since PMA, a PKC agonist, has been reported to increase sAPP_α secretion (Caporaso et al., 1992; Slack et al., 1993; Camden et al., 2005), SH-SY5Y cells were treated with PMA (10 nM) as a positive control.

Exogenous sPLA₂-IIA increased APP at the cell surface of SH-SY5Y cells

There is substantial evidence suggesting that the amyloidogenic pathway to generate Aβ occurs preferentially in the intracellular compartments, whereas the non-amyloidogenic pathway for production of sAPP_α preferentially occurs at the plasma membranes (Haass et al., 1993; Koo and Squazzo, 1994; Kinoshita et al., 2003; Small and Gandy, 2006; Cirrito et al., 2008; Rajendran et al., 2008; Schobel et al., 2008; von Arnim et al., 2008). Based on these studies, it is reasonable to hypothesize that the increase of membrane fluidity by sPLA₂-IIA favors the non-amyloidogenic pathway by lowering APP internalization and thus leading to an increase of APP at the cell surface of SH-SY5Y cells. To test this hypothesis, we fluorescently labeled the extracellular domain of APP without membrane permeabilization. Immunofluorescence microscopy showed that sPLA₂-IIA enhanced the labeling of APP at the cell surface (Fig. 5). Quantification of the fluorescent intensity indicated sPLA₂-IIA increased APP at the cell surface by ~40%.

Exogenous sPLA₂-IIA did not alter levels of total APP and α-secretase expression in SH-SY5Y cells

In order to rule out the changes in total APP and α-secretase expression being factors to enhance the release of sAPP_α, Western blot analysis was carried out after exposing SH-SY5Y cells to sPLA₂-IIA for 24 h. Results showed that sPLA₂-IIA did not alter the expressions of APP and different isoforms of α-secretases including ADAM9, ADAM10 and ADAM17 (Fig. 6A). These results showed that expressions of APP and α-secretases were not factors in the increased sAPP_α secretion in SH-SY5Y cells when exposed to sPLA₂-IIA.

Exogenous sPLA₂-IIA did not alter levels of Aβ₁₋₄₂ in SH-SY5Y cells

Since sPLA₂-IIA increased the secretion of sAPP_α in SH-SY5Y cells, it is possible that this activity may decrease the production and secretion of Aβ₁₋₄₂ in these cells. However ELISA measurements showed that the secretion and production of Aβ₁₋₄₂ from SH-SY5Y cells was not altered upon treatment with sPLA₂-IIA for 24 h (Fig. 6B, C). Furthermore, Western blot analysis showed that exposing SH-SY5Y cells to sPLA₂-IIA for 24 h did not alter the expressions of BACE1 (Fig. 6A).

DISCUSSION

In this study, we provided evidence showing the ability for pro-inflammatory cytokines (TNF_α and $\text{IL-1}\beta$) to induce $\text{sPLA}_2\text{-IIA}$ production and release to culture medium in DITNC astrocytes. We also showed the action of $\text{sPLA}_2\text{-IIA}$ to alter membrane fluidity and enhance sAPP_α secretion from neuron cells (SH-SY5Y). Studies further demonstrated that action of $\text{sPLA}_2\text{-IIA}$ was abrogated through blocking by specific inhibitor and that exogenous human recombinant $\text{sPLA}_2\text{-IIA}$ added to neuron cells also produced similar results.

Astrocytes regulate neuronal activities in a variety of ways under physiological and pathological conditions. They are providers of nutrients and recycling units for neurotransmitters. In many pathological conditions, astrocytes become reactive and some genes are upregulated, which is associated with increase in immune responses and alteration of signaling pathways leading to release of cytokines and altered response to oxidative stress and apoptotic pathways (Zamanian et al., 2012). Metabolic interactions between reactive astrocytes and neuronal cells under pathological conditions have been an important subject for investigation. $\text{sPLA}_2\text{-IIA}$ is regarded an inflammatory enzyme and increased levels in plasma have been well demonstrated in human inflammatory diseases such as arthritis (Hurt-Camejo et al., 2001). There is also recognition about the role of $\text{sPLA}_2\text{-IIA}$ in neurological disorders (Yagami et al., 2014). In our earlier studies, an increase in $\text{sPLA}_2\text{-IIA}$ was observed in astrocytes in AD brain as compared with non-demented elderly brains (Moses et al., 2006). Increase in $\text{sPLA}_2\text{-IIA}$ mRNA was also observed in the rat brain after focal cerebral ischemia (Lin et al., 2004). In studies with astrocytes, pro-inflammation cytokines were shown to increase $\text{sPLA}_2\text{-IIA}$ mRNA expression through activation of the $\text{NF-}\kappa\text{B}$ transcription pathway (Li et al., 1999; Wang and Sun, 2000; Jensen et al., 2009). $\text{sPLA}_2\text{-IIA}$ from astrocytes was linked to the production of reactive oxygen species and apoptosis through activation of the superoxide producing enzyme NADPH oxidase in neurons (Yagami et al., 2014).

Although PLA_2 are responsible for maintenance of phospholipid homeostasis and membrane properties, little is known about target action of $\text{sPLA}_2\text{-IIA}$. In our previous study, $\text{sPLA}_2\text{-III}$ from bee venom was shown to alter membrane fluidity and increase sAPP_α production in SH-SY5Y cells (Yang et al., 2010). In this study, we demonstrated that $\text{sPLA}_2\text{-IIA}$ from cytokine stimulated astrocytes regulates sAPP_α secretion in neuronal cells. Our data in DITNC astrocytes showed that $\text{sPLA}_2\text{-IIA}$ is induced by pro-inflammatory cytokines including TNF_α and $\text{IL-1}\beta$, and is secreted to the culture medium (Fig. 1). By applying the conditioned medium to SH-SY5Y cells, we mimicked the effects of astrocyte–neuron interaction in brain. The conditioned medium containing $\text{sPLA}_2\text{-IIA}$ from cytokine-stimulated DITNC astrocytes increased sAPP_α secretion and membrane fluidity in SH-SY5Y cells (Fig. 2). Addition of selective $\text{sPLA}_2\text{-IIA}$ inhibitor to the conditioned medium abrogated the effects on sAPP_α secretion and altered membrane fluidity in SH-SY5Y cells, suggesting that active $\text{sPLA}_2\text{-IIA}$ is in part responsible for these effects (Fig. 2).

Our results showed that recombinant human $\text{sPLA}_2\text{-IIA}$ also increased sAPP_α secretion and membrane fluidity in SH-SY5Y cells (Fig. 4). These results are in agreement with other

studies showing action of PLA₂s to increase membrane fluidity (Kameyama et al., 1982; Schaeffer et al., 2005) and sAPP_α secretion (Cho et al., 2006).

APP is a trans-membrane protein with cleavage sites for β- and γ-secretases to produce Aβ and for α-secretases to produce sAPP_α (Small and Gandy, 2006; Muller and Zheng, 2012; Jiang et al., 2014; Perneczky et al., 2014). APP internalization and translocation to endosomes is a key factor for Aβ production (Kinoshita et al., 2003; Small and Gandy, 2006; Cirrito et al., 2008; Rajendran et al., 2008; Schobel et al., 2008; von Arnim et al., 2008). Kojro and colleagues (2001) have demonstrated increasing cholesterol content in cell membrane to decrease APP internalization and increase sAPP_α secretion. This study also suggests that sPLA₂-IIA decreases APP internalization (Fig. 5) and increases sAPP_α secretion (Fig. 4). There is evidence that endogenous Aβ may decrease membrane fluidity and stimulate its own production, thus forming a vicious cycle (Peters et al., 2009). However, our data as well as those from Kojro et al. (2001) and Peters et al., 2009 tend to support the notion that high membrane fluidity leads to an increase in APP accumulation at the cell surface, and a subsequent increase in sAPP_α production.

α-Secretase cleavage of APP has been reported to compete and preclude the β-secretases (BACE) cleavage, the primary step for production of harmful Aβ (Haass et al., 1993; Koo and Squazzo, 1994). However, in this study, enhanced sAPP_α secretion (~ twofold) induced by sPLA₂-IIA did not result in a detectable decrease in Aβ secretion and production (Fig. 6). In fact, Kim et al. (2008) was not able to show a link between sAPP_α and Aβ production (Kim et al., 2008). Similarly, other studies have found that increased APP at the cell surface and sAPP_α production do not necessarily result in an Aβ₁₋₄₂ decrease when SH-SY5Y cells were treated with arachidonic acid (Yang et al., 2010, 2011). Certainly, further studies are needed to better understand underlying mechanism.

In agreement with the notion that AD is a chronic inflammatory disease, a number of studies indicated increased pro-inflammatory cytokines associated with the pathogenesis of this disease (Beloosesky et al., 2002; Lahiri et al., 2003; Cacquevel et al., 2004; Patel et al., 2005; Zuliani et al., 2007; Baranowska-Bik et al., 2008; Deniz-Naranjo et al., 2008; Couturier et al., 2010; Pellicano et al., 2010). For example, Aβ promotes the expression of TNF_α and IL-1β in peripheral blood mononuclear cells from AD patients (Pellicano et al., 2010). Add discussion and citations about toxic Aβ to cells. In AD transgenic mouse models, inflammatory cytokine levels correlate positively with Aβ levels and locations (Mehlhorn et al., 2000; Apelt and Schliebs, 2001; Patel et al., 2005). Our study showed the induction of sPLA₂-IIA from astrocytic DITNC cells by TNF_α and IL-1β, and its role in APP processing. The roles of other metabolic components from cytokine-stimulated astrocytic cells in AD require more investigations to obtain more comprehensive understanding of astrocytes in regulation of neuronal activities.

In summary, our results showed that cytokine-stimulated DITNC cells, through sPLA₂-IIA secretion, enhanced sAPP_α secretion in SH-SY5Y cells by increasing membrane fluidity. This study should provide insights into not only the roles of sPLA₂-IIA, but astrocytes in the regulation of APP processing in neurons.

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Abbreviations

AD	Alzheimer's disease
APP	amyloid precursor protein
Aβ	amyloid- β peptide
BACE1	β -site APP cleaving enzyme
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
FCVJ	Farnesyl-(2-carboxy-2-cyanovinyl)-julolidine
FITC	fluorescein isothiocyanate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PLA₂s	Phospholipases A ₂
PMA	Phorbol 12-myristate 13-acetate
RA	all-trans retinoic acid
sAPP_{α}	α -secretase-cleaved soluble amyloid precursor protein
sPLA₂-IIA	group IIA secretory phospholipase A ₂
sPLA₂-III	group III secretory phospholipase A ₂
TBST	Tris-buffered saline containing 0.1% (v/v) Tween 20

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Fig. 1.

Cytokine induced sPLA₂-IIA secretion in DITNC cells. DITNC cells were exposed to cytokines (TNF α and IL-1 β , 10 ng/ml) for 8 h, followed by removing cytokines and incubating in serum-free medium for another 40 h. Conditioned medium was used for Western blot analysis of sPLA₂-IIA. Data are expressed as percentages of control and mean $\pm$ SD from at least three independent experiments (*** $p < 0.001$).

Fig. 2.

SH-SY5Y cells were treated with conditioned medium containing sPLA₂-IIA from cytokine-stimulated DITNC cells. (a): The conditioned medium from non-stimulated DITNC cells (control). (b): The conditioned medium from cytokine-stimulated DITNC cells. (c): The conditioned medium from cytokine-stimulated DITNC cells supplemented with the sPLA₂-IIA inhibitor (KH064, 1 μ M). (d): The conditioned medium from non-stimulated DITNC cells supplemented with KH064. (A, upper) Representative images of SH-SY5Y cells fluorescently labeled with FCVJ with conditioned medium treatment. (Scale bar=20 μ m) (A, lower) The relative fluorescent intensity of FCVJ where a lower intensity indicates increased membrane fluidity. (B) Western blot analysis of sAPP α secretion with SH-SY5Y cells treated with the aforementioned DITNC conditioned media treatment groups. Data are expressed as percentages of control and mean $\pm$ SD from at least three independent experiments with statistical analysis performed with a one-way ANOVA, followed by *Bonferroni's* post hoc tests (* p < 0.05 when compared to the control group (a) and # p < 0.05 when compared to the conditioned media treatment group (b)).

Fig. 3.

Effects of human recombinant sPLA₂-IIA on the viability of SH-SY5Y cells. MTT assay was applied after treatment of SH-SY5Y cells with different levels of sPLA₂-IIA. Data are expressed as percentages of control and mean $\pm$ SD from at least three independent experiments (** $p < 0.01$).

Fig. 4.

(A) Effects of human recombinant sPLA₂-IIA on SH-SY5Y cell membrane fluidity. Representative images of SH-SY5Y cells fluorescently labeled with FCVJ without (upper, left) and with sPLA₂-IIA treatment (upper, middle and right). Scale bar=20 μm. (B) Effects of human recombinant sPLA₂-IIA on sAPP_α secretion in SH-SY5Y cells. After treatment of SH-SY5Y cells with sPLA₂-IIA, the culture medium was used for Western blot analysis of sAPP_α. Results showed that sPLA₂-IIA increased sAPP_α secretion to medium from SH-SY5Y cells. PMA treatment known to increase sAPP_α secretion in cells was used as a positive control. Data are expressed as percentages of control and mean ± SD from at least three independent experiments (**p* < 0.05, ***p* < 0.01).

Fig. 5.

Immunofluorescence microscopy showing effects of sPLA₂-IIA on the presence of APP at the cell surface of SH-SY5Y cells. SH-SY5Y cells were treated with human recombinant sPLA₂-IIA for 24 h and were labeled with anti-APP antibody and FITC-conjugated secondary antibody without cell permeabilization. Representative images of fluorescently labeled SH-SY5Y cells without (upper, left) and with sPLA₂-IIA treatment (upper, middle and right) showed sPLA₂-IIA increased the APP accumulation at the cell surface (lower). Scale bar=20 μ m. sPLA₂-IIA increased the APP accumulation at the cell surface (lower). Data are expressed as percentages of control and mean $\pm$ SD from at least three independent experiments (* $p < 0.05$).

Fig. 6.

(A) sPLA₂-IIA on the expressions of APP, α-secretases and BACE1 in SH-SY5Y cells.

Western blot analysis showed that sPLA₂-IIA did not alter the expressions of APP, different isoforms of α-secretases (ADAM 9, ADAM 10 and ADAM 17) and BACE1. (B) ELISA assay to quantify Aβ₁₋₄₂ production from SH-SY5Y cells treated with 0, 50 or 100 ng/ml of sPLA₂-IIA. sPLA₂-IIA did not alter Aβ₁₋₄₂ secretion from SH-SY5Y cells to culture medium. (C) sPLA₂-IIA did not alter the expression of Aβ₁₋₄₂ within SH-SY5Y cells. Data are expressed as mean ± SD from three independent experiments.